PB86-208766

Reproductive Toxicity and Lack of Dominant Lethal Effects of 2,4-Dinitrotoluene in the Male Rat

Medical Coll. of Virginia, Richmond

Prepared for

Health Effects Research Lab.
Research Triangle Park, NC

1985

U.S. Department of Commerce
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1. REPORT NO. EPA/600/J-85/413	2.	PB86-208766
4. TITLE AND SUBTITLE (Journal Version) REPRODUCTIVE TOXICITY AND LACK OF DOMINANT LETHAL EFFECTS OF 2,4-DINITROTOULENE IN THE MALE RAT		5. REPORT DATE 1985 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Richard W. Lane, Glenn Stuart Simon, Robert W. Dougherty, John L. Egle, Jr., and Joseph F. Borzelleca		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298		10. PROGRAM ELEMENT NO. CBMC1A 11. CONTRACT/GRANT NO. CR804290
12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory (HERL), Office of Research and Development (ORD), U.S. Environmental Protection Agency (USEPA), Research Triangle Park, North Carolina 27711		13. TYPE OF REPORT AND PERIOD COVERED Journal Article 14. SPONSORING AGENCY CODE EPA-600/11

15. SUPPLEMENTARY NOTES
P.O. M. Kate Smith (KICampbell), BB/TMD/HERL/USEPA (CI)
Citation: Drug and Chemical Toxicology, Vol. 8, No. 4, 265-280, August 1985

16. ABSTRACT

Adult male Sprague Dawley rats were gavaged with 2,4-dinitrotoluene (2,4-DNT) dissolved in corn oil at 0, 60, 180, or 240 mg/kg/day for five days. A single oral dose (0.5 mg/kg) of triethylenemelamine was used as a positive control. Induction of dominant lethal events was scored on the basis of early fetal deaths. At the two lower doses, no consistent changes were observed in the numbers of pre-implantation losses, implantation sites, or living or non-living fetuses. The highest dose of 2,4-DNT tested resulted in a marked decrease in the numbers of sperm-positive females (determined by microscopic examination of vaginal smears for sperm) and pregnant females. These two effects diminished in the latter weeks of mating. The low number of pregnant females at the highest dose made meaningful statistical evaluations difficult. The results indicate that 2,4-DNT does not cause dominant lethal mutations but does adversely affect reproductive performance.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS *(This Report)* UNCLASSIFIED	21. NO. OF PAGES 17
	20. SECURITY CLASS *(This page)* UNCLASSIFIED	22. PRICE

EPA Form 2220-1 (Rev. 4-77)

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EPA/600/J-85/413
JOURNAL ARTICLE

DRUG AND CHEMICAL TOXICOLOGY, 8(4), 265-280 (1985)

REPRODUCTIVE TOXICITY AND LACK OF DOMINANT LETHAL EFFECTS OF 2,4-DINITROTOLUENE IN THE MALE RAT

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ABSTRACT

Adult male Sprague Dawley rats were gavaged with 2,4-dinitrotoluene (2,4-DNT) dissolved in corn oil at 0, 60, 180, or 240 mg/kg/day for five days. A single oral dose (0.5 mg/kg) of triethylenemelamine was used as a positive control. Induction of dominant lethal events was scored on the basis of early fetal deaths. At the two lower doses, no consistent changes were observed in the numbers of pre-implantation losses, implantation sites, or living or non-living fetuses. The highest dose of 2,4-DNT tested resulted in a marked decrease in the numbers of sperm-positive females (determined by microscopic examination of vaginal smears for sperm) and pregnant females. These two effects diminished in the latter weeks of mating. The low number of pregnant females at the highest dose made meaningful statistical evaluations difficult. The results indicate that 2,4-DNT does not cause dominant lethal mutations but does adversely affect reproductive performance.

INTRODUCTION

2,4-Dinitrotoluene (2,4-DNT) is an industrial intermediate used in dye manufacturing, in the production of polyurethane foams, and as a gelatinizing and waterproofing agent in the

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production of explosives[1]. 2,4-DNT is a water contaminant found in the effluent of munitions plants[2]. Exposure to this chemical can range from high-level, acute exposures (e.g., in the workplace),[3] to low level, chronic exposures (e.g., from either the workplace or drinking water). Toxicity of the dinitrotoluenes has recently been reviewed[4].

2,4-DNT is rapidly absorbed through the intact skin, the lungs, and the gastro-intestinal mucosa of man[3,5]. Vernot et al.[6] reported the acute, oral LD_{50} of 2,4-DNT as 270 mg/kg in male rats and as 1630 mg/kg in mice. 2,4-DNT produces methemoglobinemia in rats, mice, dogs[1] and in man[5] which can lead to drowsiness, shortness of breath, and collapse. Other acute effects in human of exposure to 2,4-DNT are headache, weakness, nausea, and dizziness[5].

Subchronic exposures to 2,4-DNT have produced atrophy of the testes and aspermatogenesis, decreased muscular coordination, decreased appetite, weight loss, gliosis and demyelination in the brain, and hematopoietic disturbances in rats, mice and dogs[7-9]. Human reproductive toxicity is also suspected[10].

2,4-DNT has been reported to cause hepatocellular carcinomas[8,9] and benign skin tumors and mammary tumors[11] in rats. In mice, Short et al.[1] reported the presence of cystic renal tumors, but a NCI bioassy[11] found no evidence of carcinogenicity.

Results regarding the genetic effects of 2,4-DNT in vitro are equivocal. Several investigators have reported negative

results with the Ames test[12-15]. Positive responses were found by Hodgson et al.[16], and by Couch et al.,[15] in the TA98 and TA1535 strains. Other in vitro test systems have indicated a lack of genotoxicity[12,13,16].

Additional in vivo tests have been reported, again with apparently conflicting results. 2,4-DNT did not cause an increase in the incidence of abnormally shaped sperm in mice, nor did it increase the number of recessive spots in mice[17]. Soares and Lock[17-19] did not find any indication of dominant lethality in mice after two days of dosing (i.p. or p.o.) 250 mg/kg of 2,4-DNT (both purified and technical grade). Hodgson et al.[16] reported a significant increase in the number of dead implants/total implants in rats consuming 0.2% 2,4-DNT (approximately 90 to 100 mg/kg/day) in their diet for thirteen weeks but no further information was given.

Neither of the dominant lethal (DL) studies reported conform to the dosing protocols generally recommended nor did they report the usual DL parameters[20-21]. Due to the equivocal nature of the carcinogenicity/mutagenicity data, the suspicious chemical structure, and the prominence of 2,4-DNT in manufacturing and as a water contaminant, a standardized and thorough DL test was deemed necessary to more critically evaluate the effect of the chemical on genetic material in vivo in a species sensitive to its carcinogenic effect.

METHODS

2,4-DNT (Pfaltz and Bauer, Stamford, Connecticut) was recrystallized from diethyl ether to a constant melting point. Identity was confirmed by NMR spectroscopy.

Fifty male Sprague-Dawley rats (Flow Laboratories, Dublin, Virginia), weighing 200-225 g were randomly divided into five equal groups. One group received a daily oral dose of Mazola corn oil (10 ml/kg) for five days. Three other groups received 60, 180, or 240 mg 2,4-DNT dissolved in corn oil/kg/day for five consecutive days. These doses were selected following a range-finding study; 240 mg 2,4-DNT/kg/day for five days was the highest tolerated dose. The last group received a single oral dose of triethylenemelamine (TEM) dissolved in corn oil (0.5 mg/kg body weight).

Due to unexpected excessive deaths among the members of the high dose group, another group of rats began receiving 240 mg/kg 2,4-DNT one week after the other groups. Therefore, comparisons were made to the corn oil controls of the same calendar week, not mating week (i.e., the results of the high dose group for week 1 are compared to control results for week 2), but results are reported by week after dosing.

All animals had free access to food (Purina Rodent Chow 5001) and water throughout the experiment. Weights of the males were taken daily during the dosing period and weekly thereafter for a minimum of eleven weeks. Each male was allowed to mate

with two naive, nulliparous females for five days each week. Insemination was determined by the microscopic examination of vaginal smears for the presence of sperm (day 1 of pregnancy). The proportion of females inseminated and those that became pregnant was noted. Females were sacrificed on day 14 of gestation. The uteri and ovaries were examined for living and dead implantations and corpora lutea. Mating lasted for seven weeks except for the high dose which was extended by six weeks to look for the possible reversibility of the effects of 2,4-DNT.

The following indices were calculated:

MI (Mating Index) = (total number of females pregnant/total number of females allowed to mate) x 100

FyI (Fertility Index) = (total number of females pregnant/total number of females inseminated) x 100

CLI (Corpora Lutea Index) = total number of corpora lutea/total number of pregnant females

II (Implantation Index) = total number of implantation sites/total number of pregnant females

PILI (Preimplantation Loss Index) = (total number of corpora lutea - total number implantation sites)/total number of pregnant females

FI (Fetal Index) = total number of living fetuses/total number of pregnant females

RI (Resorption Index) = total number of fetal deaths/total number of pregnant females

DF/LF = total number of dead fetuses/total number of living fetuses

DF > 1 = total number of females with one or more dead fetuses/total number of females with zero dead fetus

DF > 2 = total number of females with two or more dead fetuses/total number of females with one or zero dead fetus

DLI = 1 - living fetuses/pregnant female (experimental) x 100 living fetuses/pregnant female (control)

The data were evaluated according to Green et al.[20], Ehling, et al.[21] and Simon et al.[22]. The statistical evaluation of MI, FyI, DF/LF, DF > 1, and DF > 2 were analyzed by using 2 x 2 contingency tables (test group vs. corn oil) for each week.[22-27] Chi squares were determined after correcting for continuity. Statistical significance was determined at the 0.05 level. CLI, II, PILI, FI, and RI were analyzed by the Kruskal-Wallis one-way analysis of variance. This non-parametric test of variance was performed using a Statistical Analysis System program on an IBM 370/158 computer. Statistical significance was set at the 0.05 level and localized by performing Dunn's approximate distribution-free multiple comparison.

RESULTS

Fifty-three percent (8/15) of the second group of rats that received 240 mg 2,4-DNT/kg/day died within two weeks of receiving the first dose. During the dosing period and the subsequent

week the high dose animals were cyanotic and lost weight, the only group to lose weight in this study. A large weight gain was seen between weeks 1 and 2 of mating which may be attributed to a return to feeding after the initial effects of 2,4-DNT subsided. No other deaths occurred in the study, but cyanosis was evident during the dosing period in the group receiving 180 mg 2,4-DNT/kg/day.

The results of 2,4-DNT and TEM administration are summarized in Table 1. No significant changes occured in any of the indices examined after dosing with 60 mg 2,4-DNT/kg/day. Three statistically significant effects were seen in the females mated to males receiving 180 mg 2,4-DNT/kg/day: PILI, week 2; MI, week 5; and CLI, week 5. A number of adverse reproductive effects were observed among the rats receiving the high dose, most noticeably the MI of weeks 1-6. The low MI made it difficult to interpret the other data for those weeks. Because of this poor mating performance, it was decided to allow this portion of the study to continue six weeks longer than the other doses. By the thirteenth week, all reproductive and DL indices has recovered and were comparable to control values. TEM produced its classical DL effects[27] for the first four weeks of mating; no effects were seen on either mating or fertility.

DISCUSSION

The results of this study indicate that 60 mg 2,4-DNT/kg/day for five days produced no adverse reproductive or DL effects. No signs of toxicity were seen other than slight cyanosis.

TABLE 1
REPRODUCTIVE DATA FOR FEMALE RATS MATED TO TREATED MALES[a]
Corn Oil Control

Week	Number Pregnant[b]	MI	FyI	CLI	II	PILI	FI	RI	DF/LF	DF≥1	DF≥2
1	6	30	66	11.67	9.83	1.83	9.83	0.00	0/59	0/6	0/6
2	14	70	88	11.93	9.86	2.07	8.93	0.93	13/125	7/7	3/11
3	10	50	83	15.70	12.90	2.80	12.40	0.50	5/124	5/5	0/10
4	14	70	88	12.64	11.14	1.50	10.43	0.71	10/146	9/5	1/13
5	18	90	100	13.28	10.94	2.33	9.78	1.17	21/176	9/9	2/16
6	15	75	100	13.00	11.60	1.40	10.93	0.67	10/164	7/8	2/13
7	18	90	100	12.00	10.94	1.06	10.44	0.50	9/188	6/12	3/15
8	13	65	93	11.69	11.15	0.54	10.69	0.46	6/139	4/9	1/12
9	15	75	100	11.27	10.07	1.20	9.73	0.33	5/146	4/11	1/14
10	16	80	100	11.88	11.25	0.63	10.50	0.75	12/168	10/6	2/14
11	16	80	94	11.75	11.00	0.75	10.31	0.69	11/165	7/9	3/13
12	13	65	87	11.69	9.62	2.08	8.31	1.31	17/108	8/5	4/9
13	14	70	100	11.93	11.07	0.86	10.29	0.79	11/144	7/7	4/10
14	18	90	100	11.28	9.50	1.78	8.89	0.61	11/160	7/11	1/17

TEM (0.5 mg/kg)

Week	Number Pregnant[b]	MI	FyI	CLI	II	PILI	FI	RI	DF/LF	DF≥1	DF≥2	DLI
1	8	40	80	11.50	8.13	3.38	4.25	3.88[c]	31/34[c]	8/0[c]	6/2[c]	56.77
2	11	55	92	14.09	10.00	4.09	4.82	5.18[c]	57/53[c]	10/1	9/2[c]	46.02
3	12	60	80	13.67	8.75	4.92	2.83[c]	5.92[c]	71/34[c]	12/0[c]	9/3[c]	77.18
4	16	80	100	12.81	10.56	2.25	8.44	2.13	34/135[c]	10/6	7/9	19.08
5	16	80	100	12.31	11.25	1.06	10.56	0.69	11/169	8/8	3/13	-7.98
6	16	80	94	12.81	11.69	1.13	11.25	0.44	7/180	6/10	1/15	-2.93
7	14	70	100	12.54	11.23	1.31	10.38	0.85	11/135	8/6	1/13	0.57

2,4-DNT (60 mg/kg)

Week	Number Pregnant[b]	MI	FyI	CLI	II	PILI	FI	RI	DF/LF	DF≥1	DF≥2	DLI
1	8	40	100	14.75	12.25	2.50	12.00	0.25	2/96	2/6	0/8	-22.08
2	14	70	100	13.57	11.86	1.71	11.21	0.64	9/157	7/7	2/12	-25.53
3	12	60	86	14.92	10.67	4.25	10.00	0.67	8/120	6/6	1/11	19.35
4	15	75	100	12.47	10.60	1.87	10.20	0.40	6/153	4/11	1/14	2.21
5	18	90	100	12.44	11.44	1.00	10.28	1.17	21/185	9/9	1/17	-5.11
6	14	70	93	13.14	10.57	2.57	10.07	0.50	7/141	6/8	1/13	7.87
7	15	75	100	12.07	10.53	1.53	10.07	0.47	7/151	4/11	3/12	3.54

2,4-DNT (180 mg/kg)

Week	Number Pregnant[b]	MI	FyI	CLI	II	PILI	FI	RI	DF/LF	DF≥1	DF≥2	DLI
1	6	30	75	12.33	9.00	3.33	8.17	0.83	5/49	3/3	1/5	16.89
2	9	45	75	15.11	9.22	6.00[c]	7.89	1.33	12/71	5/4	3/6	11/65
3	8	40	80	13.13	11.50	1.63	11.00	0.50	4/88	4/4	0/8	11.29
4	9	45	75	13.11	11.33	1.78	10.89	0.44	4/98	4/5	0/9	-4.41
5	11	55[c]	85	9.82[c]	8.45	1.36	7.73	0.73	9/85	5/6	1/10	20.96
6	15	75	83	11.93	11.93	0.80	10.73	0.40	6/161	4/11	1/14	1.83
7	14	70	93	12.36	10.93	1.43	10.14	0.79	11/142	3/11	3/11	2.87

(Table 1 Cont.)

(Table 1 Cont.)

2,4-DNT (240 mg/kg)

Week	Number Pregnant[b]	MI	FyI	CLI	II	PILI	FI	PI	DF/LF	DF≥1	DF≥2	DLI
1	4	20[c]	100	11.00	6.00	5.00	4.75	1.25	5/19	3/1	1/3	46.81
2	3	21	60	12.33	5.67	6.67	5.00	0.67	2/15	2/1	0/3	59.68
3	1	7[c]	50	8.00	3.00	5.00	0.00	3.00	3/0[c]	1/0	1/0	100.00
4	2	14[c]	100	7.00	1.50	5.50	0.00	1.50	3/0[c]	2/0	1/1	100.00
5	1	7[c]	100	7.00	1.00	6.00	0.00	1.00	1/0	1/0	0/1	100.00
6	2	14[c]	68	8.00	3.00	5.00	2.00	1.00	2/4[c]	2/0	0/2	80.84
7	5	36	71	11.60	5.00[c]	6.60[c]	5.00[c]	0.00	0/25	0/5	0/5	53.23
8	6	43	68	13.50[c]	10.50	3.00[c]	10.00	0.50	3/60	3/3	0/6	-2.77
9	6	43	86	13.00	11.17	1.83[c]	10.17	1.00	6/61	3/3	2/4	3.14
10	8	57	100	11.88	10.25	1.63	9.38	0.88	7/75	6/2	1/7	9.02
11	9	64	90	9.78	7.11	2.67	6.67	0.44	4/60	4/5	0/9	19.74
12	9	64	82	10.56	9.44	1.11	9.22	0.22	2/83	2/7	0/9	10.40
13	10	71	91	11.80	10.90	0.90	10.50	0.40	4/105	3/7	1/9	-18.11

[a] Corn oil and 2,4-DNT: 5 daily oral doses; TEM: 1 oral dose in corn oil
[b] 20 females mated per week
[c] $p \leq 0.05$

The next dose used, 180 mg 2,4-DNT/kg/day for five days, produced cyanosis, but only three (out of 77) reproductive or DL values were statistically different from corresponding control values. The MI (week 5) which was statistically significantly lower than the controls occurred at the same time that the MI of the control group reached its highest value. Also, the MI values for this dose of 2,4-DNT increased steadily throughout the seven weeks of mating. It is concluded that although the MI may be slightly affected by 2,4-DNT at a dose level of 180 mg/kg/day, the effect is temporary. The lone significant CLI change (week 5) cannot be attributed to 2,4-DNT, but rather to biological variation. The increased PILI (week 2) could be due to 2,4-DNT, but is probably due to biological variation. No overall pattern was observed which could be construed as 2,4-DNT causing dominant lethality at this dose level.

Weight loss, cyanosis, and death occurred during the week after dosing male rats with 240 mg 2,4-DNT/kg/day. All indices that were tabulated were affected, MI and DF/LF most severely. Although the FyI was reduced for most of the study, no statistically significant differences were obtained. This finding contrasts with that of Soares and Lock[20], who reported a significant reduction in male mouse fertility at a comparable dose (250 mg/kg/day for two days). Most of the changes observed during the first six weeks were probably due to the effects of 2,4-DNT on male reproductive function and are not specifically

DL effects. The low number of pregnant females made statistical analysis of the results difficult to interpret.

After week 7 of the study (i.e., the extended portion), the MI had recovered sufficiently (i.e., was not significantly different from control) to allow the other data to be more easily interpreted. II, PILI and FI were significantly altered in week 7. Thereafter, only the PILI of weeks 8 and 9 were statistically significant from control. It should be noted that these indices were different from control values before week 7, but not at a statistically significant level. However, the spectrum of effects was not similar to that produced by the positive control (TEM). By week 11, there appeared to be little difference between the data from treated and control animals. It is therefore concluded that 240 mg 2,4-DNT/kg/day for five days has major reproductive effects in the rats and that these effects are slowly reversible.

Additional tests such as sperm counts, motility, and viability need to be done after chronic and/or multigeneration exposures to 2,4-DNT. Detailed histopathology of the testes would also prove useful in trying to determine the most sensitive cells of the spermatogenic cycle. Binding of radiolabelled 2,4-DNT to the DNA of sperm or testicular DNA repair could be useful in assessing the genotoxicity of 2,4-DNT.

It is concluded that in the rat there are no DL effects caused by 2,4-DNT. There are only moderate, and reversible,

effects on MI and FI at 180 mg 2,4-DNT/kg/day and no adverse reproductive effects at 60 mg/kg/day. Severe reproductive effects were seen after five days of dosing at 240 mg 2,4-DNT/kg. These effects persisted for at least eight weeks (approximately one spermatogenic cycle in the rat). Recovery from the toxic effects of the high dose of 2,4-DNT was complete by the end of two spermatogenic cycles. Although the long period of reproductive toxicity at this dose is of concern, these toxicities are evident only at a dose which is severely debilitating and are not indicative of a dominant lethal effect.

ACKNOWLEDGEMENTS

This study was supported in part by EPA Grant No. R-804290010.

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